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Cyclic CMP Alters Squirrel Monkey (*Saimiri sciureus*) Luteal Cell Structure via Cyclic AMP-Dependent Mechanisms

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ABSTRACT—Cyclic cytidine monophosphate (cCMP) is a pyrimidine nucleotide that is not as well-known as the other intracellular messengers such as cyclic adenosine monophosphate (cAMP). The present study was undertaken to determine if cCMP functions to alter cultured luteal cell morphology and if the mechanism involves cAMP. Adult female squirrel monkeys of Guyanan origin were administered daily single i.m. injections of FSH (follicle-stimulating hormone) for 4 days followed by hCG (human chorionic gonadotropin) on the fourth day. Luteal cells were aspirated from ovarian corpora lutea 64 hr after hCG by laparoscopy. The cells were dispersed, washed and cultured in individual center-well petri dishes for 48 hr at 37°C in 5% CO_2 in air. The test compounds (10 μM each), dbcCMP, dbcAMP, dbcGMP, dbcCMP and 6 mM imidazole, or control media were added at the start of culture. At the end of the incubation period, the cells were fixed in methanol and stained with Giemsa stain. The data indicated that dbcCMP restructured the polygonal luteal cells into small narrow-shaped cells with minimal cytoplasm in a manner similar to dbcAMP. However, when imidazole, a phosphodiesterase stimulator, was also present, the cells retained the polygonal shape. Dibutyryl cGMP at the concentration tested did not affect luteal cell morphology. The results suggest that dbcCMP alters the squirrel monkey luteal cell morphology in a manner similar to dbcAMP and that the process requires intracellular cAMP.

INTRODUCTION

Cyclic nucleotides are a class of intracellular compounds [1–3] which are involved in mediating hormone action and regulating cellular events such as the assembly of microtubules [4, 5] and microfilament cell processes [6]. The role of cAMP and cGMP as intracellular second messengers is well-documented [2, 3, 7, 8].

At the present time, there have been no reports on the function of cCMP in the regulation of cell morphology and progesterone synthesis. Cyclic CMP is a pyrimidine compound, in contrast to cAMP and cGMP which are purine compounds. Studies in other cell types indicate that cCMP activates protein kinases [9], hemoglobin synthesis [10], modulates cell proliferation and increases phosphodiesterase activity in fast growing hepatoma cells [11]. The present study was carried out to define the action of cCMP, in relation to cAMP and cGMP, on squirrel monkey luteal cell morphology. The objective is to determine if cCMP alters cell morphology and to examine the mechanism involved in the transformation.

MATERIALS AND METHODS

Adult female squirrel monkeys (*Saimiri sciureus*) of Guyanan origin (Buckshire Corp., Perkasie, PA) weighing between 600 g and 750 g were housed individually in stainless steel flush-type cages. The animals were kept indoors on a 12L:12D cycle and fed a commercial monkey feed and water *ad libitum*. The hormonal-stimulating regimen [12] consisted of administering the female monkeys with 4 days of follicle-stimulating hormone (FSH-P, Burns-Biotec Laboratories Inc., Omaha, NE) at a daily dosage of 1 mg intramuscu-

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larly followed by a single i.m. injection of 250 IU human chorionic gonadotropin (hCG, APL Ayerst Laboratories, New York, NY) on the final day of FSH treatment. It has been shown that there is no seasonal effect on squirrel monkey oocyte development and that follicle cells such as luteal cells may be harvested at any time of the year [13]. At 64 hr after hCG treatment, the squirrel monkeys were anesthetized (15 mg ketamine/animal i.m.) and laparoscoped. The luteal cells were aspirated from the corpora lutea in the ovaries using a 1 ml tuberculin syringe fitted with a 25 gauge needle. The cells were dispersed into the culture medium, gently minced, washed and pipetted into the center well of individual Falcon #3037 petri dishes and incubated as low-density cultures at 37°C in a moist atmosphere of 5% CO_2 in air. The test compounds, 10 μM each of dbcCMP, dbcAMP, dbcGMP, dbcCMP plus 6 mM imidazole or control media were added at the start of culture. After 48 hr of incubation, the cells were fixed in methanol and stained with Giemsa stain [14]. Photomicrographs of the stained cells in the different treatments were taken and analyzed using the Zeiss Videoplan computerized image analyzer as described below.

Culture medium

The culture medium consisted of Medium 199 with 25 mM HEPES buffer, Earle's salts and L-glutamine (GIBCO, Grand Island, New York) and 75 mg/l penicillin and 75 mg/l streptomycin. The medium was filtered through a 0.22 micron syringe filter (Nalgene Co., Rochester, NY) and supplemented with 20% filtered and heat-inactivated fetal bovine serum (HyClone, Logan, UT).

Videoplan computerized image analysis

Morphometric measurements of the photomicrographs of the cells were quantitated using the Zeiss Videoplan computerized image analyzer equipped with statistical software. The image analyzer operated by translating the movements of the tracing of the outline of selected two-dimensional objects using an electronic input pen placed on a magnetized tabloid. The area and perimeter of the nucleus and individual cell outline were measured. The data collected were expressed as mean $\pm$ S. E. For convenience, the units of measurement were in arbitrary units. The actual perimeter in microns may be calculated by multiplying each value by 0.2545. In the case of the area value, multiplying each area value by 0.0648 should provide the actual value in square microns.

Statistical analysis

Differences in the perimeter and area measurements were tested for significance using the Dunnett's t-test for comparisons with the control with heterogeneous variance and unequal treatment sample sizes. A $P<0.05$ value was considered significant.

RESULTS

The presence of 10 μM dbcCMP in the media resulted in a significant reduction ($P<0.01$) in the cross-sectional area of the squirrel monkey luteal cell (Table 1). The cross-sectional area of the nucleus of the dbcCMP-treated cell was also significantly smaller in comparison with the control cell nucleus. When the cAMP-specific phosphodiesterase stimulator, imidazole, was present along with dbcCMP, the cells did not show the characteristic reduction in cell shape. Light microscopic observations of the cells revealed differences in shape of dbcCMP-treated and control luteal cells (Fig. 1). The control cells were polygonal-shaped while the dbcCMP-treated cells were small and narrow-shaped with less cytoplasm. The dbcCMP cells also appeared to have less pseudopod formation and less granular cytoplasm compared with the control cells. Some of the dbcCMP-treated cells had a wide spindle-shaped appearance. We did not observe any other cell types along with the cultured luteal cells. When imidazole was present along with dbcCMP (Fig. 2), the luteal cell morphology was similar to the control cell morphology and did not have the small narrow-shaped appearance characteristic of dbcCMP-treated cells.

The luteal cells became smaller and narrow-shaped when 10 μM dbcAMP was added to the culture media (Fig. 3). The decrease in cell shape and structure was significant ($P<0.001$) in comparison with the control (Table 1). The cross-

TABLE 1. The effect of cyclic nucleotides on the morphology of squirrel monkey (*Saimiri sciureus*) luteal cells *in vitro*

Treatment	Mean area of cell nucleus (sq. units)	Mean area of entire cell (sq. units)
Control	73.7±4.6 (27)	628.5±36.2 (22)
10 μM dbcCMP	58.6±4.0 (28)[a]	451.0±34.6 (33)[b]
10 μM dbcCMP+ 6 mM imidazole	75.6±2.3 (32)	603.3±50.7 (22)
10 μM dbcAMP	52.0±4.0 (33)[b]	396.6±25.1 (26)[b]
10 μM dbcGMP	73.0±3.7 (26)	701.6±42.4 (23)

[a] Significant difference from respective control ($P<0.05$).
[b] Significant difference from respective control ($P<0.01$).
Values are expressed as mean±S.E. (sq. arbitrary units).
Values in parentheses indicate number of cells.

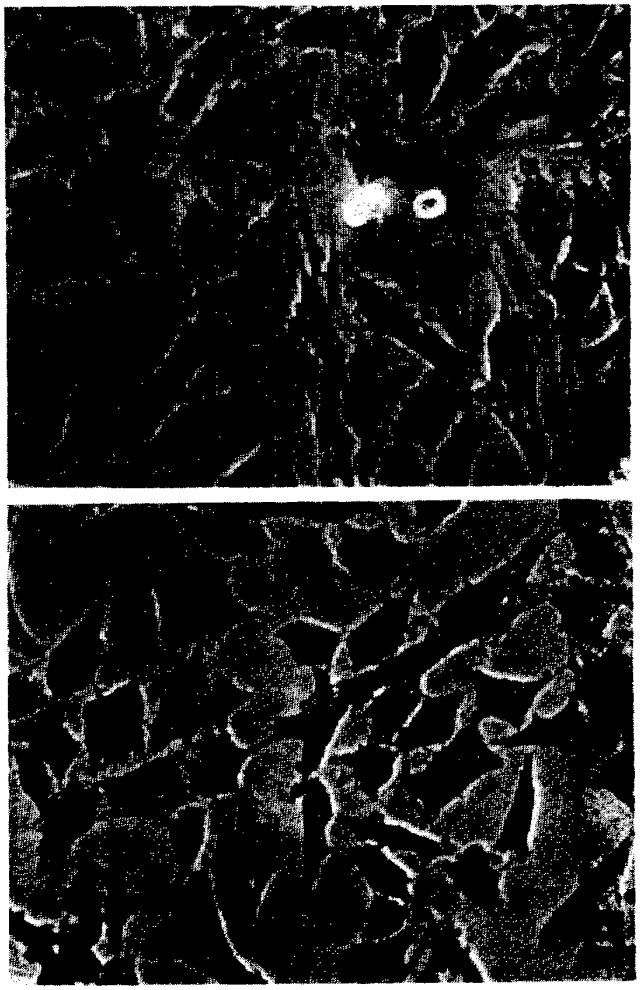

FIG. 1. The morphology of squirrel monkey luteal cells growing in low-density cultures after 48 hr of incubation in either control media (A) or in the presence of 10 μM dbcCMP (B). Note the decreased cell size and reduced cytoplasm in the dbcCMP-treated cells. Magnification ×400, phase contrast.

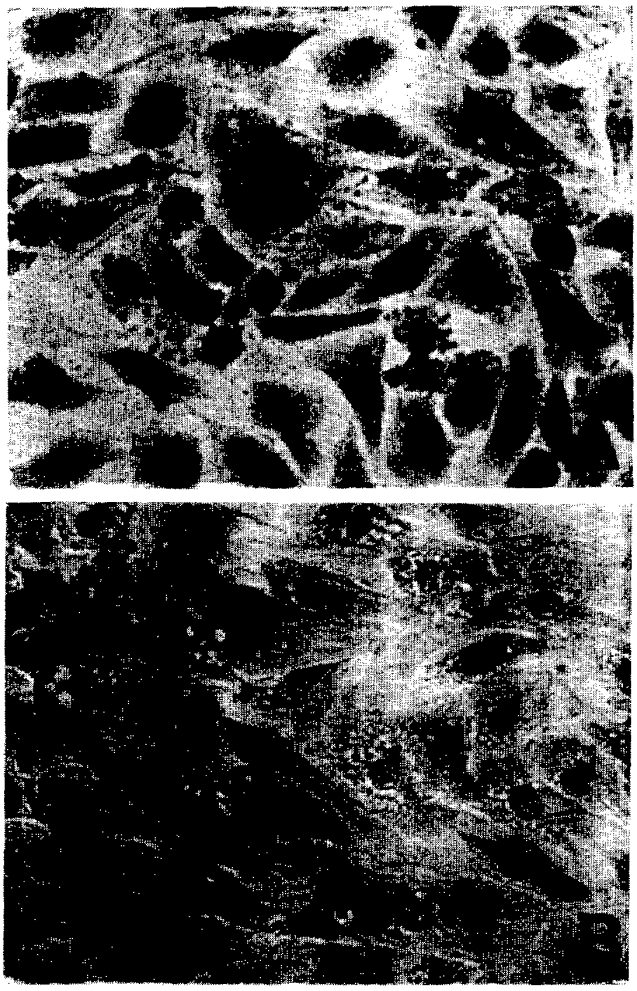

FIG. 2. Two views of squirrel monkey luteal cells in low-density cultures after 48 hr of incubation in the presence of 10 μM dbcCMP and 6 mM imidazole; light microscopy (A) and phase contrast (B). Magnification ×400.

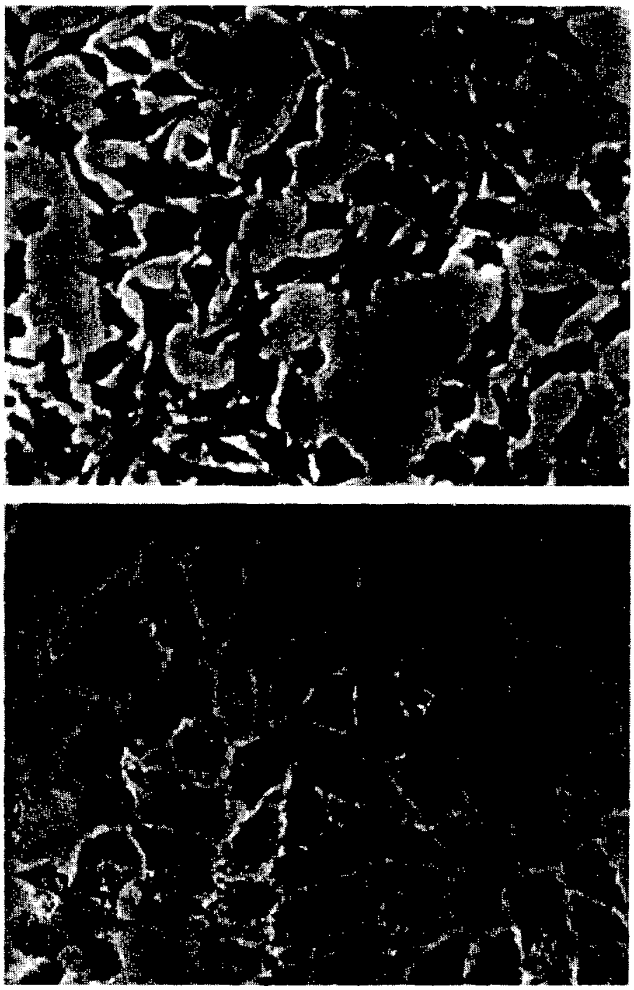

FIG. 3. The effect of 10 μM dbcAMP (A) or 10 μM dbcGMP (B) on the morphology of squirrel monkey luteal cells in low-density cultures after 48 hr of incubation. Magnification $\times$400, phase contrast.

sectional area of the cell nucleus was also decreased by the dbcAMP treatment. Some of the cells showed a pronounced spindling effect. In contrast, the addition of 10 μM dbcGMP to the cultures did not have an effect on cell morphology (Fig. 3). An analysis of the cross-sectional area of the dbcGMP-treated luteal cells also did not reveal any significant differences from control cells (Table 1).

DISCUSSION

This report is the first demonstration of *in vitro* cultured squirrel monkey (*Saimiri sciureus*) luteinized granulosa cells or luteal cells. In the present study, we have demonstrated that the action of dbcCMP on the alteration of the morphology of cultured squirrel monkey luteal cells is similar to the action of dbcAMP on these cells. This suggests that the function of cCMP may be similar to cAMP. The size of the luteal cell soma and nucleus are both decreased by dbcCMP and dbcAMP treatment. Our observation that dbcAMP alters the luteal cell morphology is supported by studies showing the same effect in other cell types such as fibroblasts [6, 15, 16] and granulosa cells [17, 18]. The alteration in cell size by cAMP and by other compounds that increase intracellular cAMP such as luteinizing hormone (LH) has been correlated to active steroidogenesis in the cell [17] and may involve reorganization of the tubulin and intermediate filaments [18, 19] through calmodulin-mediated mechanisms [20]. We surmise that cCMP may be involved in the control of steroidogenesis in luteal cells because of the similarity of dbcCMP-treated cells and dbcAMP-treated cells. The synthesis of progesterone by the squirrel monkey luteal cells will be examined in a future experiment.

There is very little information on the role of cCMP in the cell. Cyclic CMP is a pyrimidine compound, unlike cAMP and cGMP which are purine compounds. A related compund, cytidine-3′-monophosphate is a ribonuclease inhibitor and it is possible that cCMP may also function as such. The role of cCMP in other cell activities such as stimulating cancer cell proliferation has been reported [21, 22]. A study done on pigeon crop-sac mucosal epithelial cells suggests a role of cCMP as a mediator in the proliferation of cells [23]. The activity of cCMP-phosphodiesterase (PDE) has been shown to be high in slow growing hepatoma cells and low in rapidly-dividing cells [24] and the cCMP-PDE appears to be a separate and different PDE from the cAMP-specific PDE [25]. Another study suggested a role of cCMP in stimulating hemoglobin synthesis [10] probably via protein kinases [9]. In this study we postulate a role of cCMP in the control of steroidogenesis in luteal cells.

The action of dbcCMP on squirrel monkey luteal cells can be inhibited by imidazole, a cAMP-specific PDE stimulator implying that the mechanism of action of dbcCMP requires intracellular cAMP. We cannot exclude the possibil-

ity that the imidazole may be stimulating PDE to inactivate both intracellular cAMP and exogenous dbcCMP, although this argument is weak because it has been shown that dibutyryl cyclic nucleotide analogues are resistant to degradation by PDE [26]. It is possible that the role of cCMP is to be a mediator or co-factor for cAMP-induced cell shape changes, calcium-mediated events and steroidogenesis.

In this study, we also tested dbcGMP because certain concentrations of cGMP appear to affect intracellular cAMP production [27]. Our data indicated that dbcGMP was not involved in the alteration of luteal cell morphology. It appears that cGMP does not play a role in the physiological processes in the squirrel monkey luteal cell.

The results of the present study suggest that cCMP alters the squirrel monkey luteal cell morphology in a manner similar to cAMP and that the process requires intracellular cAMP.

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